REMARKS

Claims 1, 3, 6 and 7 are pending in the application. Claims 1 and 3 are amended. Applicants reserve the right to pursue any withdrawn or canceled subject matter in one or more continuation or divisional applications.

Rejections under 35 U.S.C. §112

The Examiner has rejected claims 1-7 under 35 U.S.C. §112 second paragraph as indefinite because it is allegedly unclear to whom the compound is being administered. The claims have been amended to recite that the compound is administered to "a human patient in need thereof" to overcome this rejection.

The Examiner has also rejected claims 1-7 for the recitation of the phrase "optionally substituted." It would be clear to one skilled in the art that the residue of an amino acid, as described on page 5, lines 17-26, could be optionally substituted.

The Examiner has also rejected claims 1-7 stating that it is not clear whether Ala, Val, Leu or Ile represents a substitution on another amino acid or a different substitution for R1. The claims have been amended to clarify that residue R1 is, alternatively, a residue that is "derived from Ile."

Prior Art Rejections under 35 U.S.C. §§ 102 and 103

The Examiner has rejected claims 1-7 under 35 U.S.C. §102(b) and under 35 U.S.C. §103(a) over Vandai (U.S. 5,212,158).

The '158 patent discloses compounds falling under formula (I) of present claim 1 and the alleged *nootropic* effect of these compounds (see page 2, line 6). However, Vandai fails to disclose the suitability of these compounds for the treatment of postlesional diseases

characterized by nerve cell necrosis due to ischemia, trauma or intoxication, as recited in the amended claims.

Vandai does not disclose or suggest a mechanism of action of the compounds described therein would indicate any suitability for use in postlesional diseases characterized by nerve cell necrosis, as recited in the amended claims. As stated above, Vandai discloses the alleged *nootropic* effects of the desribed compounds.

A specific mechanism of action of the nootropics is not known. While at first an improvement of the blood flow in the brain as well as an improvement of the cerebral glucose utilization was assumed to be more or less a pharmaco-therapeutic mechanism of action, in the '80s and '90s an improvement of the cerebral energy metabolism or even a direct influence on the central nervous transmitter was discussed more often (see for example the publication by Professor Jean Rapin, one of the inventors, in *La Lettre du Pharmacologue*, Volume 6, No. 6, May 1992, copy enclosed as Exhibit A). In detail the following mechanisms for various nootropics were explained: the increase of the metabolism rate of nerve cells (Piracetam, Pyritinol), the blockage of the damaging calcium inflow into the nerve cells (Nimodipin), protection against excessive release of messengers (Memantine) and neutralization of cell-damaging metabolism products (ginkgo biloba).

Furthermore, the alleged disclosure of treatment of neurodegenerative diseases in Vandai does not anticipate or render obvious the amended claims to the treatment of postlesional diseases. The promotion of neurite growth – which is a prerequisite for the treatment of postlesional diseases of necrotic origin – does not belong to one of the possible mechanisms of action known from the literature, and has no connection whatsoever with the mechanism of action known for nootropics.

A postlesional disease of toxic origin, as recited in the amended claims, is *not* the same a neurodegenerative disease and *does not* encompass Alzheimer's disease or amnesia, as recited in Vandai. A postlesional disease of toxic origin is induced by exogenous toxins such as alcohol,

drugs, heavy metals, etc. β-amyloid protein plaques are generated only within the central nervous system and are thus endogenous toxins, rather than exogenous ones. β-amyloid also cannot be administered to the central nervous system to induce toxicity, for example, via the blood circulation, since it does not pass the blood-brain barrier and is metabolized peripherally. In support of this, Alzheimer's disease and postlesional diseases of toxic origin are differently classified by the World Health Organization. Alzheimer's is classified in block 30 of the International Statistical Classification of Diseases and Related Health Problems (see www.who..int/whosis/icd10/) while postlesional diseases of toxic origin (i.e. intoxication) are classified as 'injury' in block S. The claimed methods would not have been obvious to one skilled in the art in view of Vandai.

Similarly, amnesia is not a postlesional disease of traumatic origin. Amnesia is not even a specific disease, but instead is a *symptom*. Amnesia is not associated with (necrotic) cell death so that its treatment does not require nerve regeneration, but instead it is a reversible cognitive impairment.

It is thus not surprising that in the pertinent literature a difference is drawn between a therapeutic treatment of neurodegenerative conditions, for which nootropics are used, such as for example Alzheimer's disease, and *regenerative* processes which are necessary for the treatment of postlesional diseases of the nervous system, as presently claimed (see Varon and Connor (1994) "Nerve Growth Effector in CNS Repair" in *Journal of Neurotrauma*, vol. 11, no. 5, Exhibit B, attached). On page 473, Varon et al. state:

> In fact, NTFs are already being evaluated in clinical trials as potential therapeutic agents for major human neurodegenerative conditions, such as Alzheimer's, Parkinson's, and motor neuron diseases (Olson et al., 1992; Seiger et al., 1993). Much less attention, on the other hand, has been given to the involvement of neurotrophic factors in CNS regenerative processes (for a recent review, see Varon and Hagg, 1993). [emphasis added]

This reference supports the contention that the nootropic or even anti-neurodegenerative effect of special substances do not render their effect on regenerative processes obvious. The

present application contains experimental data proving the neuro-regenerative effect of the compound Cinnamoyl-GFPNH$_2$. This data supports the neuro-regenerative effect of the claimed compounds. There is no suggestion in Vandai that the compounds recited in the amended claims are regenerative. There is therefore no suggestion in Vandai that these compounds could be used in a method of treatment of postlesional diseases characterized by necrotic cell death, as recited in the amended claims, which require such regeneration.

Double Patenting

The Examiner has provisionally rejected claims 1-7 under the judicially created doctrine of obviousness-type double patenting over claims 1-7 of copending Application no. 10/635,696. The Examiner has apparently equated the treatment of postlesional diseases claimed in the pending application with the treatment of neurodegenerative diseases claimed in the '696 application.

Contrary to the Examiner's assertion, these claims do not cover overlapping subject matter. A postlesional disease, as recited in the claims, is *not* the same a neurodegenerative disease. A postlesional disease of toxic origin is induced by exogenous toxins such as alcohol, drugs, heavy metals, etc. As noted above, Alzheimer's disease and postlesional diseases of toxic origin are differently classified by the World Health Organization. Alzheimer's is classified in block 30 of the International Statistical Classification of Diseases and Related Health Problems (see www.who..int/whosis/icd10/) while postlesional diseases of toxic origin (i.e. intoxication) are classified as 'injury' in block S. A skilled person therefore can clearly distinguish Alzheimer's disease form a postlesional diseases of toxic origin.

Further, amnesia is not considered a postlesional disease of traumatic origin. Amnesia is not a specific disease, but instead is a *symptom*. Amnesia is not associated with (necrotic) cell death so that its treatment does not require nerve regeneration, but instead it is a reversible cognitive impairment.

Applicants believe no further fees are due with this response, however if the Examiner determines that any fees are due, the Commissioner is hereby authorized to charge any additional fees associated with this response to Deposit Account No. 11-0980.

Respectfully submitted,

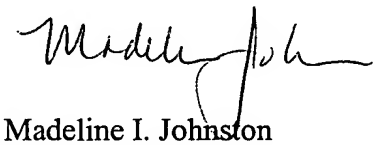

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EXHIBIT A

Les nootropes : propriétés pharmacologiques du piracétam et indications thérapeutiques

• J.-R. Rapin*

RÉSUMÉ. *La définition d'une substance nootrope comme un activateur cognitif repose en fait sur une amélioration des communications membranaires et plus particulièrement neuronales, sans faire intervenir directement les neuromédiateurs ou leurs récepteurs. Dans ces conditions les effets pharmacologiques sont observés dans des cas pathologiques (hypoxie, électrochoc...) et uniquement sur les fonctions d'intégration comme l'apprentissage et la mémorisation. De ces propriétés découlent les indications du piracétam, seul nootrope commercialisé, proposé pour améliorer certains symptômes du déficit intellectuel du sujet âgé (attention, mémoire), proposé dans le traitement des infarctus cérébraux constitués, des vertiges d'origine centrale et comme traitement d'appoint de la dyslexie chez l'enfant.*

Mots clés : *Nootropes - Piracétam - Apprentissage - Mémoire - Communication interneuronale - Plasticité cellulaire.*

Le concept nootrope, proposé par Giurgea [1], repose sur le rôle particulier du télencéphale dans l'activité nerveuse supérieure comme centre d'intégration des fonctions cognitives et mentales. Les nootropes constituent une classe de psychotropes qui visent directement, sans modification de la vigilance réticulo-limbique, à faciliter l'efficience de l'activité intégrative du cerveau. Ce concept est fondé sur l'organisation fonctionnelle du cerveau, proposée par Luria, en trois unités :
— une unité d'entrée, de codage et de stockage des informations provenant des mondes extérieur et intérieur (partie postérieure du cerveau et aires motrices) ;
— une unité de vigilance et d'homéostasie (système réticulaire et limbique) ;
— une unité de programmation et de contrôle du comportement complexe (télencéphale).

Tous les psychotropes connus, à l'exception du lithium, agissent directement sur l'unité de vigilance et d'homéostase, c'est-à-dire qu'ils entraînent des effets soit sédatifs, soit stimulants. Un nootrope agit au niveau du télencéphale et améliore l'efficience des connections interhémisphériques. Selon la classification des psychotropes de l'OMS, un nootrope n'est ni neuroleptique, ni anxiosédatif, ni antidépresseur, ni psychostimulant. A la différence des médicaments de ces classes, un nootrope n'agit pas par l'intermédiaire des neuromédiateurs.
Les nootropes ne se fixent pas sur des récepteurs cérébraux et n'interviennent pas sur les processus de libération ou de recapture des médiateurs. Leur action porte sur la membrane plasmique permettant de meilleures communications intracellulaires et cette action ne se limite pas aux seuls neurones, d'où des effets en hémorrhéologie sur les plaquettes et sur les autres tissus.

Le piracétam, seul nootrope actuellement commercialisé, est un dérivé cyclique du GABA *(fig.1)*. D'autres molécules de structure chimique proche du GABA répondent au concept nootrope. De plus, il est possible de rattacher à ce groupe de substances des dérivés dont les propriétés reconnues portent sur la plasticité des membranes cellulaires.

PROPRIÉTÉS PHARMACOLOGIQUES

Les propriétés pharmacologiques du piracétam ont été démontrées chez un grand nombre d'espèces animales comprenant les souris, rats, chats, lapins, poissons, et portent sur la facilitation des apprentissages et de la mémoire et aussi sur les agressions telles que l'hypoxie, l'électrochoc et l'intoxication par les barbituriques.

Protection de l'activité cérébrale contre les agressions

Action antihypoxique

La protection cérébrale contre les agressions a été démontrée en évaluant l'activité électrique corticale et les capacités d'apprentissage des animaux. Ainsi, les rats entraînés à un test d'évitement passif et soumis à des conditions d'hypoxie donnent, lorsqu'ils sont traités par du piracétam, une meilleure réponse de rappel. Le piracétam supprime donc les effets amnésiques de l'hypoxie. Nikolova et coll. [2] élargissent le spectre antihypoxique du piracétam à d'autres modèles compre-

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nant les hémorragies, les ischémies et les anoxies. En relation avec l'activité comportementale, l'administration de piracétam permet un retour à un EEG normal après une période d'anoxie chez le lapin et le rat. Les effets antihypoxiques du piracétam ont aussi été démontrés par la potentialisation des actions de la prostacycline et des dérivés de l'ergot [3].

Action sur la dépression corticale
Le piracétam réduit la dépression corticale induite par le chlorure de potassium, l'adénosine monophosphate et le phénobarbital. Puisque ces substances ont des mécanismes de dépression locale différents, il est raisonnable de penser que le piracétam exerce un effet non spécifique sur les fonctions neuronales résultant d'une protection et de la maintenance de l'activité corticale.

Facilitation de l'apprentissage et de la mémoire
Les effets bénéfiques du piracétam sur l'apprentissage et la mémoire ont été démontrés chez différentes espèces (souris, rats, poissons) à l'aide de nombreux tests d'acquisition et de mémorisation faisant intervenir des évitements actifs, passifs et des rappels [4].

Sur la rétention, les capacités sont chez le rat de l'ordre de 100 % et il n'est pas possible d'obtenir une amélioration. En revanche, il est classique de provoquer des amnésies par divers stimuli, ce qui revient aux effets anti-agressions déjà décrits. Comme exemple prenons un évitement passif chez le rat. Quel que soit le critère utilisé, les rats normaux montrent une très bonne rétention 24 heures après l'acquisition. Si en revanche les rats sont soumis tout de suite après l'acquisition à l'action d'un agent amnésiant (électrochoc, hypoxie...) on constate que l'agent amnésiant a empêché la consolidation de cette acquisition ou a empêché une évocation normale de ce type de mémoire. Le piracétam protège contre l'amnésie et la rétention mnésique est quasi normale *(fig. 2)*.
L'activité du piracétam est différente sur la mémorisation en fonction de l'âge des animaux. Si l'effet est significatif chez l'animal jeune, il est beaucoup moins important que chez l'animal âgé [5]. Ce résultat correspond à une action plus importante du piracétam chaque fois qu'il existe une pathologie, le vieillissement pouvant être considéré comme une forme de pathologie.

Facilitation des connexions interhémisphériques
La stimulation électrique du gyrus suprasylvien médian provoque sur la zone controlatérale du cortex cérébral un potentiel évoqué. Cette réponse est dite transcalleuse parce qu'elle n'apparaît pas chez l'animal dont le corps calleux est sectionné. L'injection de piracétam augmente l'amplitude de la transmission interhémisphérique. Une autre démonstration de cette facilitation du transfert transcalleux est mise en évidence par un apprentissage mono-oculaire. L'information arrive premièrement à l'hémisphère opposé à l'œil découvert. La trace mnésique doit passer d'un hémisphère à l'autre par l'intermédiaire du corps calleux. Le piracétam augmente la vitesse de transfert entre les deux hémisphères.

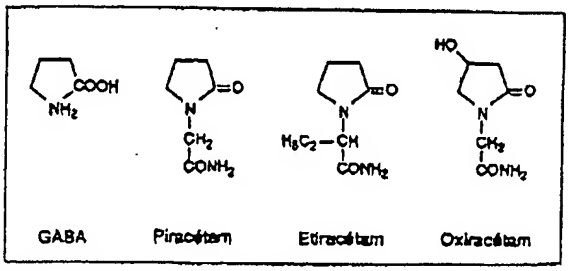

FIG. 1. *GABA et dérivés nootropes.*

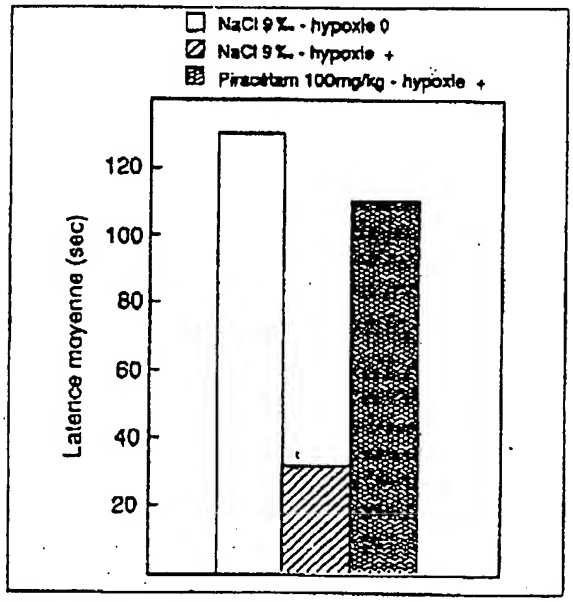

FIG. 2. *Evitement passif et hypoxie.*

Efficience du contrôle inhibiteur cortico-sous-cortical
Le cortex exerce une activité de nature inhibitrice sur les structures sous-corticales. Avec l'âge, et surtout lors des vieillissements pathologiques, le contrôle cortical s'affaiblit avec apparition de désinhibition. Chez l'animal, le modèle utilisé est celui du nystagmus central. Une stimulation du corps genouillé latéral entraîne un nystagmus stable. Le piracétam supprime ce nystagmus. Des résultats équivalents ont été trouvés avec le nystagmus vestibulaire. L'ensemble des informations prouve que le piracétam renforce les activités corticales et permet une reprise du contrôle sous-cortical.

Effet sur la microcirculation
Le piracétam améliore la microcirculation sous certaines conditions. Cet effet a été observé aussi bien au niveau central que périphérique. Le débit sanguin cérébral n'est pas modifié chez l'animal vigile et dépourvu de pathologie. A l'inverse, un accroissement du débit est observé au niveau des zones périischémiques ou lorsque l'apport est insuffisant.

TABLEAU I - *Effets et indications du piracétam*

1. ACTION CELLULAIRE : Augmentation de la plasticité

2. ACTION FONCTIONNELLE

Neurones et glie	Plaquettes	Erythrocytes
Communication interneurone et interneuroglial Communication interhémisphérique	agrégabilité adhésion	déformabilité adhésion agrégabilité

3. INDICATIONS : proposé

— pour améliorer certains symptômes du déficit intellectuel du sujet âgé (attention, mémoire...)
— dans le traitement des infarctus cérébraux constitués
— dans les vertiges d'origine centrale
— dans le traitement d'appoint de la dyslexie chez l'enfant

Plasticité neuronale

Nous pouvons considérer deux types de plasticité neuronale :
— la mise en place d'un réseau de substitution lors d'une altération d'une fonction ;
— la repousse dendritique, si le corps du neurone a conservé son potentiel de régénération.

Au cours du vieillissement, une diminution du nombre de neurones dans les aires corticales et les noyaux de la base est observée. Au niveau moléculaire, la membrane plasmique s'épaissit, perd sa fluidité avec en particulier une augmentation de la teneur en cholestérol et une modification de la composition en phospholipides. Le nombre de récepteurs de membrane diminue ainsi que la quantité de neurotransmetteurs libérés. Dans l'ensemble, les neurones sont moins plastiques. Ceci se traduit par une diminution des fonctions supérieures telles que la mémoire, la vigilance et le comportement. Au cours de la dyslexie, le dysfonctionnement frappe l'hémisphère dominant avec une diminution des relations interneuronales. Il s'agit ici encore d'une diminution de la plasticité neuronale.

Plasticité érythrocytaire et plaquettaire

Pour assurer son rôle de transporteur d'oxygène, le globule rouge doit se déformer afin de passer à travers les capillaires dont le diamètre est inférieur. Cette déformabilité dépend de la concentration en ATP et de la composition en phospholipides. De plus, la déformabilité permet un contact et, par conséquent, une diffusion de l'oxygène du globule rouge vers l'endothélium du capillaire. Le piracétam, à dose thérapeutique, entraîne une meilleure déformabilité des érythrocytes et diminue la formation de rouleaux.

Les plaquettes jouent un rôle dans l'hémostase, la thrombose et l'artériosclérose. Ces rôles sont exacerbés lors d'une diminution de la teneur en AMPc avec comme conséquence une hyperagrégabilité et une perte des phénomènes de rétrocontrôle ne permettant pas de stopper le phénomène d'induction. Ici encore il s'agit d'une perte de plasticité qui est inversée par le traitement par le piracétam en raison sans doute d'un accroissement de la synthèse en AMPc.

MÉCANISME D'ACTION DU PIRACÉTAM

Toutes les actions du piracétam, aussi bien centrales que périphériques, peuvent s'expliquer par des effets membranaires.

Le piracétam et la stucture phospholipidique membranaire

Le piracétam assure une régulation de la structure phospholipidique de la membrane en accélérant le turn over des dérivés phosphorylés. Ainsi, l'incorporation du phosphore radioactif dans les phospholipides de la membrane est augmentée sous l'action du piracétam et ceci est observé aussi bien au niveau des neurones, des érythrocytes que des plaquettes. De plus, au niveau des membranes microsomiales, le piracétam accroît, in vivo, la synthèse de phosphatidylcholine de 52 % et celle de phosphatidyléthanolamine de 80 %. Enfin, le métabolisme des phospholipides est régulé sous l'action du piracétam avec une réduction de production de glycérolphospho-éthanolamine et de phosphosérine. Ces phénomènes sont en faveur d'une réduction de la dégradation des molécules indispensables à l'intégrité des membranes plasmiques.

Le piracétam et l'équipement membranaire destiné à la communication cellulaire

Dans l'équipement membranaire, nous pouvons distinguer les récepteurs spécifiques, les canaux ioniques et les mécanismes d'exocytose permettant la libération des médiateurs. Ces mécanismes font intervenir des constituants protéiques dont la synthèse est augmentée par un traitement par le piracétam. De plus, le piracétam accroît le nombre de récepteurs disponibles en particulier au niveau du cortex : par ailleurs, le piracétam augmente le taux d'AMPc au niveau des cellules et enfin entraîne une accélération de la libération de l'acétylcholine, de la dopamine et de la noradrénaline en phase post-hypoxique, phénomène relevant de l'activation de l'exocytose et du rôle neuromodulateur du piracétam. La résultante de ces actions permet une meilleure communication cellulaire.

Plasticité cellulaire

Les mécanismes d'action évoqués ci-dessus, ainsi que les résultats observés aussi bien au niveau neuronal qu'au niveau de

cellules circulantes, nous amènent à proposer un mécanisme unique définissant l'action du piracétam. Cette substance améliore la plasticité cellulaire, c'est-à-dire la capacité d'une cellule de modifier sa réactivité en changeant de forme ou de fonction. La plasticité est incluse dans la traduction du message reçu par le cellule, dans le tri et le stockage de l'information, dans la capacité d'adapter la réponse et dans la capacité d'intégration de nouveaux circuits. Ainsi, dans le vieillissement, la diminution de la fluidité de la membrane neuronale entraînerait une diminution du nombre de récepteurs, une diminution de synthèse des neuromédiateurs et du second messager et une diminution des stratégies compensatoires d'où l'apparition de symptômes cliniques. Il semble que le piracétam puisse intervenir sur ces phénomènes par une action indirecte sur la plasticité.

« RATIONNEL » DE L'UTILISATION DU PIRACÉTAM

Le *tableau I* résume les effets et les indications du piracétam. Les indications thérapeutiques du piracétam sont en fait une extrapolation des données expérimentales. Les études cliniques récentes montrent que ce composé apporte un bénéfice thérapeutique limité [6,7] sans relation avec les espérances pharmacologiques. De nombreuses études cliniques menées en double insu dans les conditions strictes qui sont actuellement reconnues pourront peut-être étendre les champs d'application thérapeutique du piracétam et des composés nootropes. En particulier, le traitement des myoclonies postanoxiques par le piracétam montre des résultats encourageants.

CONCLUSION

Du concept nootrope qui correspond à l'activation des zones corticales d'intégration lorsque celles-ci sont déficitaires, le piracétam est devenu le chef de file d'une nouvelle classe thérapeutique agissant sur la plasticité cellulaire. Une meilleure plasticité aura pour conséquences un accroissement des interrelations neuronales et une facilitation des communications interhémisphériques. Cet effet neurotrope proprement dit est associé aux effets hémorrhéologiques observés. ■

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RÉUNIONS ET CONGRÈS

Le dossier clinique et ses rapports.
Paris, 11 juin 1992, Espace Moncassin.
Informations : Dr J.F. Henry. Médicament et Santé, Hôpital Broca, 54-56, rue Pascal, 75013 Paris. Tél. : 49 60 75 70. Fax : 49 60 10 92.

L'IFIP *organise à Paris le 11 juin 1992 :* « **Les POS selon les BPC** », *les 22-23 juin 1992 :* « **Gestion des données des essais cliniques** », *le 24 juin 1992 :* « **Présentation et communication des résultats d'essais cliniques** ». Informations : IFIP, BP 244, 92108 Boulogne cedex. Tél. : 46 03 38 80.

3e Journées de prévention des infections nosocomiales.
Paris, 25-26 juin 1992.
Inscriptions : GK Congrès, 4, rue Jean-Maridor, 75015 Paris. Tél. : 45 57 96 47. Fax : 45 58 26 97.

First international symposium on imidazoline preferring receptors.
Paris, June 29-30 1992, Maison de la Chimie, 28, rue Saint-Dominique, 75007 Paris.
Informations : ARCUS A.V., 3, rue Rousselet, 75007 Paris. Tél. : 45 67 68 01. Fax : 45 67 17 88.

GASTRONOMIE

Les essais en ouvert d'Alex Corton.
Il est des rues que l'on ne voit pas, même en les croisant sans cesse, chemins déserts, venelles improbables, voies fréquentées par leurs seuls habitants. La rue Saint-Nicolas appartient à ce monde du banal, seulement empruntée par ceux du meuble en gros. Elle se contente d'être bitumée, entre deux trottoirs, bordée d'immeubles anonymes. On y pénètre par hasard, lorsqu'une manifestation place de la Bastille crée la thrombose, l'obstacle à contourner au plus vite. Personne, hors les familiers, ne sait qu'au 7 existe le restaurant Antoine, autrement dénommé Chez Marcel.
Le dernier effort du susdit, en matière d'aménagement, remonte aux années cinquante, lorsqu'il a décidé d'éclairer son restaurant par des abat-jour Liberty. Les tables sont dans un alignement immuable, campées sur leurs pieds de fonte. Les banquettes, de moleskine, sombre bien entendu. Le bar près de la porte, comme le veut la tradition des limonadiers. On plante, d'entrée de jeu et sans barguigner devant les convives, la bouteille de crème de cassis, le bourgogne aligoté, afin de leur permettre de doser le kir selon leur coutume personnelle. Avec un pot de beaujolais, à boire pur ou discrètement relevé d'un doigt de cassis, mélange appelé, en fonction de l'orientation politique, un communard ou un cardinal.
Ensuite le jeu est ouvert, dans la profusion. Entrée - plat du jour - fromage - dessert - café. Entrée : cochonnailles ? Les terrines, les saucissons secs, les rillettes jusqu'à suffisance. J'y ai rencontré un petit salé directement tiré de la légende, servi à l'assiette, accompagné d'une saucisse fumée sublime et de lentilles, tandis que sur la desserte la dame du lieu plaçait l'excédent. Exactement portion identique, 100 % de rab. Si d'aventure une place reste pour le fromage, la maison ne fait pas dans la mesquinerie. Puis riz au lait crème anglaise comme le préparait ma mère-grand. L'ensemble pour une addition réellement sociale. Chipoteux et bec d'oiseau s'abstiendront, régime light de même, sous peine d'attirer l'attention et d'être évacués par le Samu. Les cinéphiles évoqueront les films Front populaire. Les affamés prendront trois kilos.

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EXHIBIT B

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Nerve Growth Factor in CNS Repair

SILVIO VARON and JAMES M. CONNER

ABSTRACT

The hypothesis that neurotrophic factors play important roles in the adult central nervous system (CNS) has been successfully investigated in the past decade with regard to experimental and pathologic situations. Trophic roles in adult CNS axonal regeneration, on the other hand, have received much less attention. We review three groups of recent studies that demonstrate the relevance of nerve growth factor (NGF) for the regeneration of selected axons into adult central nervous tissue. The first group concerns a septohippocampal model where transected septal cholinergic axons are allowed to regrow into the hippocampal formation through a peripheral nerve bridge implanted into the transection lesion gap. NGF is required in the bridge, enhances penetration of the hippocampal tissue when infused there, and both attracts and promotes sprouting within the septum when infused in the lateral ventricle or the septal tissue itself. The second group of studies concerns the development of a spinal cord sensory regeneration model, where dorsal root ganglionic axons regrow into a nerve bridge placed within the dorsal spinal cord. Preliminary data indicate that NGF infusion rostral to the bridge once again promotes substantial penetration of the adult cord tissue by the regenerating NGF-sensitive fibers. In the third group of studies, attention has been shifted to the location of endogenous NGF in the adult rat hippocampal formation and the normal or lesion-induced occurrence of extrasomal NGF immunoreactivity. These regions of anchored NGF have the ability to attract NGF-sensitive growing axons and may provide opportunities to investigate local cues for final definition of terminal fields.

INTRODUCTION

NEUROTROPHIC FACTORS (NTFs), already well recognized for their important functions on developing neurons of the peripheral nervous system (PNS), were proposed in the mid-1980s to play additional and equally important roles in the central nervous system (CNS) of adult mammals (e.g., Appel, 1981; Varon et al., 1982, 1984; Hefti et al., 1989). Since then, the ability of trophic factors to prevent or reduce degenerative responses of adult mammalian CNS neurons to a variety of injuries has been firmly established in experimental animals (Hefti, 1986; Williams et al., 1986; Kromer, 1987; Sievers, et al., 1987; Anderson et al., 1988; Carmignoto et al., 1989; Otto and Unsicker, 1990; Hyman et al., 1991; Pezzoli et al., 1991; Chadi et al., 1993; Hagg and Varon, 1993b; Ventrella, 1993). In fact, NTFs are already being evaluated in clinical trials as potential therapeutic agents for major human neurodegenerative conditions, such as Alzheimer's, Parkinson's, and motor neuron diseases (Olson et al., 1992; Seiger et al., 1993). Much less attention, on the other hand, has been given to the involvement of neurotrophic factors in CNS regenerative processes (for a recent review, see Varon

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and Hagg, 1993). In the present communication, we review three recent investigations of nerve growth factor (NGF) that document its competence as a regulator of adult rat intracentral axonal regeneration, namely (1) the regeneration of septal cholinergic axons into the hippocampal formation, (2) the regeneration of central sensory axons from the dorsal root ganglionic neurons into the spinal cord, and (3) the occurrence of endogenous NGF in extrasomal locations with the potential to serve as guidance signals for intrahippocampal reinnervation.

NGF AND THE SEPTOHIPPOCAMPAL CHOLINERGIC REGENERATION MODEL

Medial septum (and diagonal band) cholinergic neurons project to the hippocampal formation (HF) mainly via the fimbria-fornix tract. A complete aspirative transection of the fimbria-fornix deprives the HF of its cholinergic afferents, in addition to depriving the septal cholinergic neurons of their HF trophic contributions and causing demonstrable damage to many medial septum cholinergic (MSC) neurons (Hagg et al., 1988). Even under intraventricular administration of exogenous NGF, MSC axons could not regrow across the lesion-induced cavity unless an appropriate bridge was implanted into it. Several different bridge materials have been found competent to solicit or permit outgrowth of cholinergic fibers from the septum (Kromer et al., 1981; Wendt, 1985; Tuszynski et al., 1990). We have chosen to use a segment of peripheral sciatic nerve and to quantify the progression of regenerating cholinergic septal fibers through and beyond the nerve bridge by counting the number of fibers crossing imaginary lines at the hippocampal end of the bridge, the entrance of the HF, and 1 mm, 2 mm, or 3 mm into the HF itself (Fig. 1A). Cholinergic fibers invaded the nerve bridge to reach a maximal number by 1 month, but their entry into the HF was much slower and decreased with a sharp progression beyond the first 1–2 mm of hippocampal tissue (Fig. 1B). The resistance of adult CNS tissue to penetration by adult CNS axons (at least in this model) appeared to occur with a spatial/temporal gradient away from the lesion rather than as a spatially defined barrier (Hagg et al., 1990b).

Our trophic hypothesis (Hagg et al., 1993) proposed that the cholinergic axonal regeneration would require NGF not only at the nerve cell body level but also along the bridge and in the innervation target territory. The nerve bridge could be viewed as a mechanochemical scaffold (axially arrayed tunnels of laminin-coated basal lamina) plus an NTF-producing population of living cells (Schwann cells, fibroblasts). Elimination of the living cells (by repeated freeze-thawing and subsequent debris phagocytosis) yielded acellular nerve segments, which had lost their competence as cholinergic regeneration bridges but which regained it on preincubation with exogenous NGF (Hagg et al., 1990a). When exogenous NGF was infused into the medial septum, cholinergic axons invading a fresh nerve bridge were greatly reduced, whereas massive cholinergic sprouting was induced toward or within the regions of NGF administration (Hagg and Varon, 1993a). These observations confirmed the neurite-promoting competence of NGF in vivo and also demonstrated a tropic action of NGF (i.e., a directional guidance related to NGF sites and gradients). It was also confirmed that these neuritic effects of NGF were displayed only in the septum ipsilateral to the lesion (i.e., by axotomized MSC neurons). Finally, infusion of exogenous NGF directly into the hippocampal formation led to an earlier and more substantial penetration of regenerating cholinergic fibers into the adult hippocampal tissue, thereby compensating for, or overcoming, the natural adult CNS resistance (Hagg et al., 1990c).

Two important features have emerged from these septohippocampal model studies. One is that neurotrophic factors can participate in CNS axonal regeneration, at least in the case of one factor (NGF) and one responsive neuronal population (MSC neurons). The second feature is that NGF also has tropic effects, and, thus, the location of exogenous or endogenous NGF may be critical for determining the direction of regenerating axons. No attempts were made to address the additional question of the final distribution of regenerating axons and the local cues that may control it.

NGF AND THE SPINAL CORD SENSORY REGENERATION MODEL

A major task for future investigations is to generalize the evidence for NTF participation in CNS regeneration by demonstrating (1) the regeneration competence of NGF toward neurons other than the MSC ones and (2) the regeneration competence of neurotrophic factors other than NGF. Either approach will require the es-

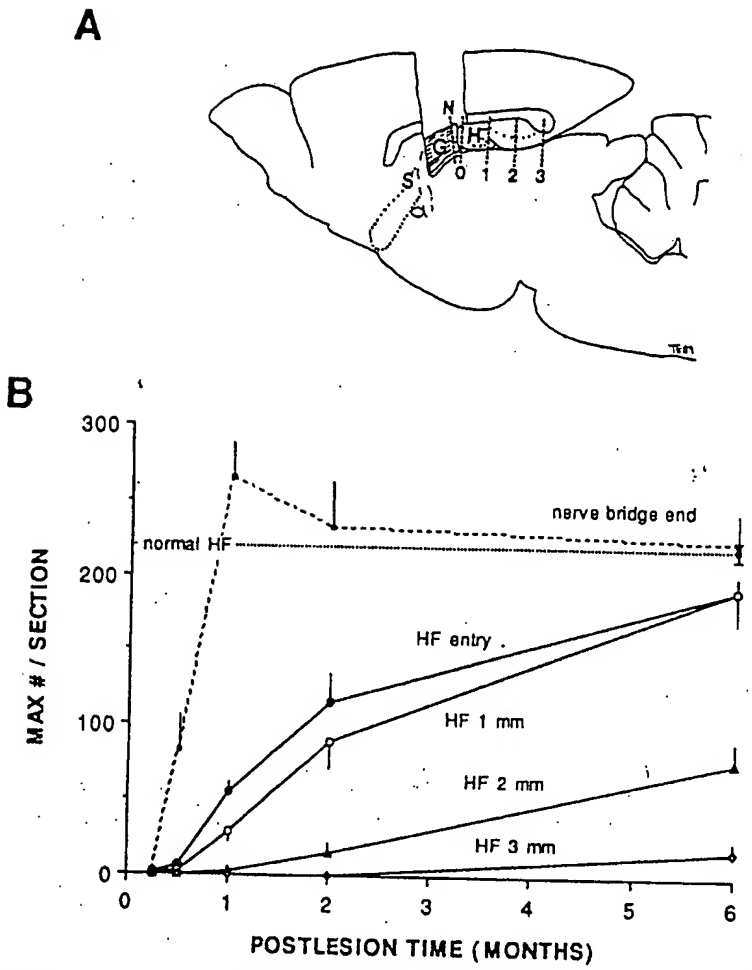

FIG. 1. The septohippocampal cholinergic regeneration model. A. Sagittal diagram indicates the oriented nerve bridge (G) grafted between septum (S) and hippocampal formation (H) and the imaginary lines at the bridge end (N), the HF entrance (O), and at various depths within the HF (1 mm, 2 mm, 3 mm), the intersections of which by AChE-stained fibers permit quantitation of the regeneration. B. Time course of the axonal advances to the bridge end and into the hippocampal tissue.

tablishment and/or use of quantifiable adult CNS models and, specifically, the definition of (1) the lesion and bridge implantation modalities, (2) the selective identification of the axons under study (by either natural or experimental labels) and their quantification, (3) baseline and time-course studies of spontaneous regeneration events, and, eventually (4) modalities and effects of exogenous NTF administrations. We have addressed these several tasks with regard to potential effects of NGF toward the intraspinal regeneration of sensory dorsal root ganglionic (DRG) neurons (Oudega et al., 1993; 1994a; 1994b; see also Fernandez et al., 1990).

The new model is illustrated in Figure 2. For a central sensory axotomy, a small segment of the dorsal funiculus (1–2 mm long, 1 mm wide, 1.5 mm deep) was excised from the midline in the T10 region. The excised tissue was replaced with a longitudinally oriented graft of peroneal nerve tissue. In some experiments, the graft material was collected from the distal segment of a peroneal nerve that had been transected 1 week before, to provide a predegenerated graft. In other experiments, the peripheral projections of lumbar DRGs into both tibial and peroneal nerves were unilaterally transected 1 day or 1 week before the central lesion and grafting to provide a conditioning lesion to the lumbar DRG neurons. Three days before the end of each experiment, trans-

ganglionic labeling of the central sensory fibers was carried out by injecting choleratoxin B subunit (CTB) into the ipsilateral sciatic nerve (Fig. 2B). Longitudinal sections of the cord were immunostained for CTB, and the number of CTB-positive fibers was determined in successive caudorostral levels along every other section. These levels (A to G in Fig. 2C) correspond, respectively, to the dorsal funiculus caudal to the lesion (A), a caudal transition zone (B), the peroneal graft (C, D, E), a rostral transition zone (F), and the cord tissue rostral to the graft (G). Special precautions were required to minimize secondary lesion effects contributing to the caudal and rostral transition zones so as to achieve satisfactory and reproducible fusion of the host cord and the peroneal graft.

In the basal conditions (fresh graft, no DRG conditioning, no exogenous NGF), several CTB-positive sensory fibers were observed coursing through the caudal transition zone toward the graft. Many of them entered the peroneal graft, but only a few emerged into the rostral transition zone, and practically none continued beyond the latter into the spinal cord itself (Fig. 3A). The use of predegenerated nerve grafts did not significantly improve this performance (Fig. 3C). In contrast, a conditioning lesion 1 week before cord lesion and graft led to an impressive increase of CTB-positive fibers throughout the pathway sequence (Fig. 3B), and a further enhancement was achieved when DRG conditioning and predegeneration of the graft were combined (Fig. 3D).

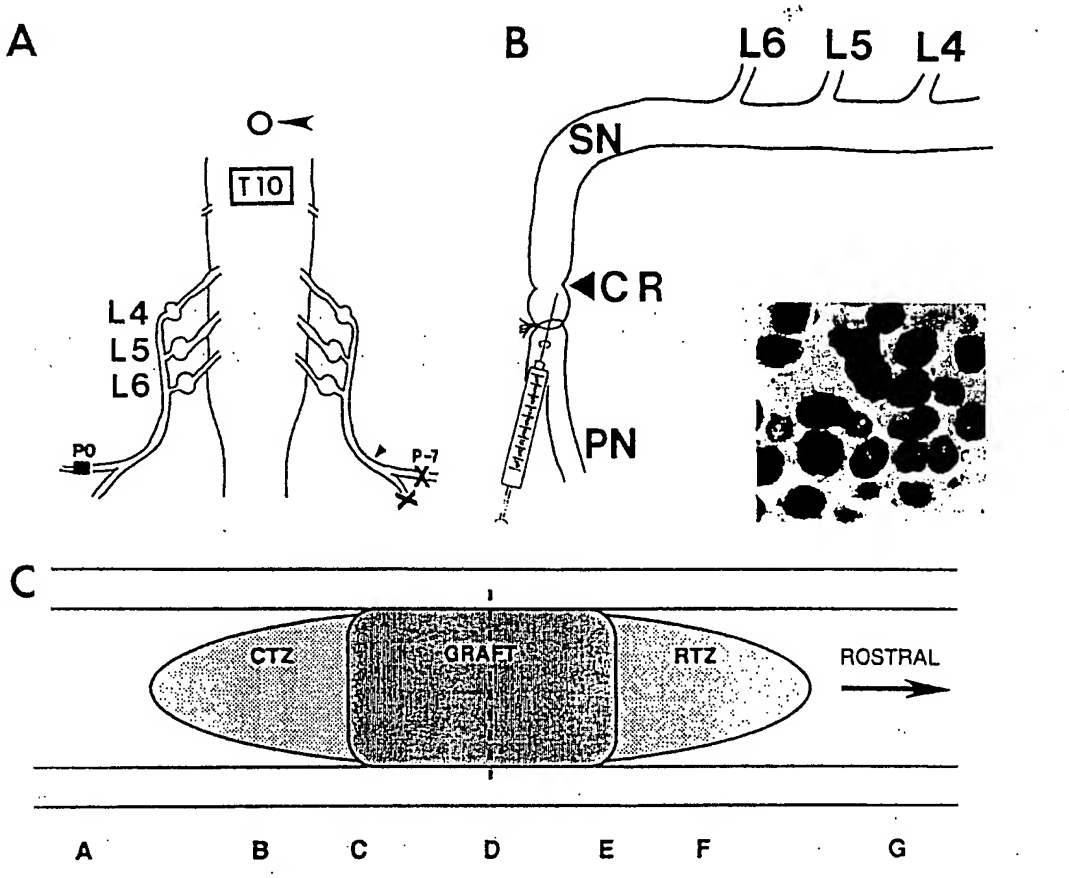

FIG. 2. The spinal cord sensory regeneration model. A. Schematic of the spinal cord with the three relevant dorsal root ganglia (L4, L5, L6), the approximate location of central lesion and nerve bridge (T10), and the more rostral location for intracord infusions (O, at arrow). P0, P7, optimal times for collection of the peroneal graft material or a conditioning lesion or both. B. Injection of choleratoxin B (CTB) tracer through the tibial nerve to the site of a crush lesion (CR) of the sciatic nerve (SN). Insert shows immunostaining of the CTB tracer in ganglionic neurons. C. Longitudinal diagram illustrating sequential levels (A to G) along the spinal cord where regenerating CTB-positive fibers are to be counted. CTZ, RTZ, caudal and rostral transition zones on either side of the graft.

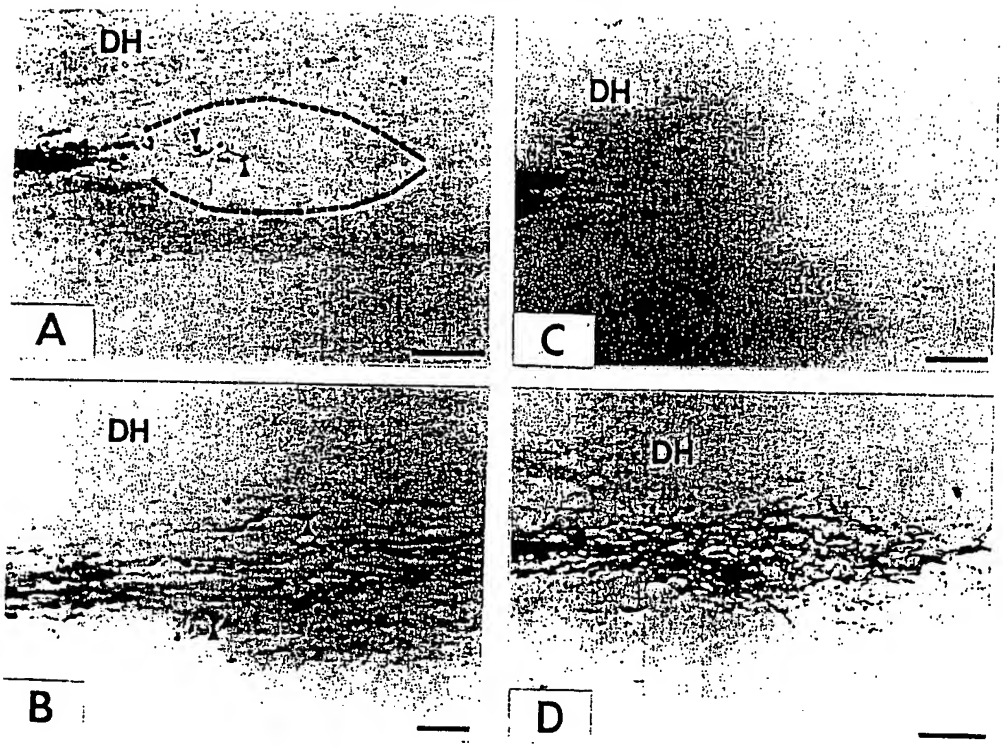

FIG. 3. DRG conditioning and graft predegeneration effects on sensory regeneration. CTB immunostaining of longitudinal cord sections. A. In the basal conditions, sensory fibers barely enter the graft (broken outline). B. Much greater penetration is achieved with a 1 week preconditioning lesion. C,D. Unconditioned (C) and conditioned (D) outgrowth into predegenerated nerve bridges. Bars = 200 μm.

A 1 day conditioning had similar but clearly lesser effects (not shown). A quantitative view of sensory axonal regeneration under those several conditions, 1 month after cord lesion and implantation, is provided in Figure 4A (fresh graft) and B (predegenerated graft). Note that 1 week conditioning coupled with a predegenerated graft (curve VI in Fig. 4B) allowed all the fibers present caudally to the caudal transition zone (level A) to reach the rostral end of the graft (level E), but still only a negligible number of them entered the cord tissue (levels F and G). A time-course study indicated that a maximal number of fibers at the graft rostral end was achieved by the end of the first week and retained throughout the first month postlesion (with partial retraction becoming apparent by the end of the second month). An important concept highlighted by these studies is that even axonal regeneration into and through a nerve graft may be potentiated or even require special manipulations (neuronal conditioning, graft predegeneration) for maximal performance. Although such requirements were compelling with regard to the adult PNS sensory DRG axons, they could still apply (perhaps less stringently) to adult CNS axons as well.

We have just begun to examine the impact of exogenous NGF in this spinal cord sensory regeneration model, using the optimized protocol of a 1 week DRG conditioning and a 1 week predegenerated peroneal graft (Oudega et al., 1993, 1994b). At grafting time, the metal cannula end of a continuous infusion device (Vahlsing et al., 1989) was inserted into the dorsal funiculus, 3 mm rostral to the rostral end of the graft and 2 mm below the dorsal cord surface. The rats were then infused (at 0.2 μL/h) with either vehicle alone or vehicle containing purified mouse β-NGF (1 μg or 10^6 trophic units per day) for 17 days (with a CTB injection 3 days before the end). Vehicle-infused animals displayed minimal outgrowth of fibers beyond the graft, as previously seen in noninfused animals (5% of the graft rostral end fibers—level E—had entered the cord tissue by 0.5 mm and no farther). Preliminary analyses (schematically depicted in Fig. 5) have indicated that, in marked contrast to vehicle infusion, infusion of mouse β-NGF dramatically enhanced sensory fiber elongation into

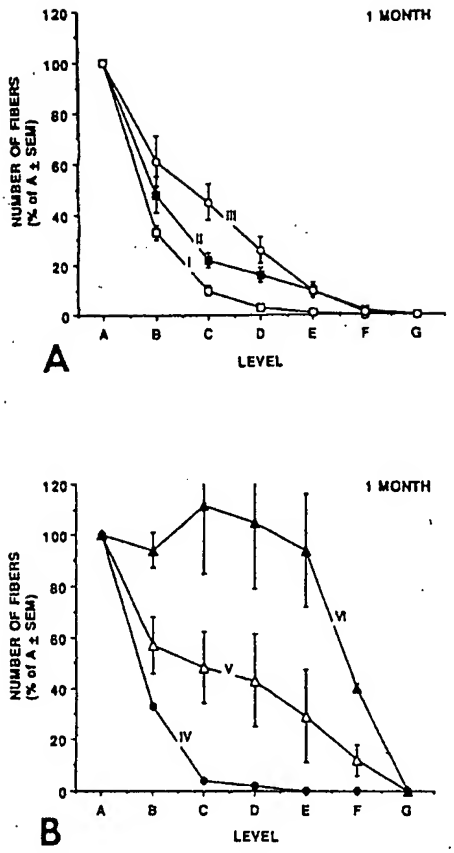

FIG. 4. Quantitative analyses of sensory fiber advances. CTB-positive fibers were counted at the various sequential levels (see Fig. 2B), using either fresh bridges (A) or predegenerated bridges (B). I and IV, no preconditioning. II and V, 1 day preconditioning. III and VI, 1 week preconditioning.

host cord tissue. Not all sensory fibers available at the rostral end of the graft were attracted into the cord by the NGF infusion, a possible reflection that only about half the dorsal root ganglionic neurons are believed to be sensitive to NGF in the adult rat (Verge et al., 1989). Interestingly, these initial observations suggest that the number of sensory fibers in the cord decreases as they grow away from the graft and closer to the NGF-infusing cannula, a possible indication of the limited time available for axonal regrowth in this set of experiments.

The new spinal cord sensory regeneration model has provided initial evidence that the NGF competence to promote in vivo CNS axonal regeneration is not unique for cholinergic neurons of the medial septum but is likely to apply to intracentral elongation of any NGF-sensitive adult axons. Whether other NTFs have similar competences for their respective target neurons remains an open question.

ENDOGENOUS NGF AND ITS DISTRIBUTION IN THE HIPPOCAMPAL FORMATION

Axonal regeneration is, of course, only part of the process through which interrupted neural connections eventually may be functionally restored. Although progress is being made on intracentral axonal regeneration—as described in the preceding sections and in recent work pertaining to oligodendroglial-related inhibitors (Schnell and Schwab, 1990; Cadelli and Schwab, 1991)—the final location and functional competence of the new axonal terminals remain to be addressed, as do the molecular properties of CNS tissues that

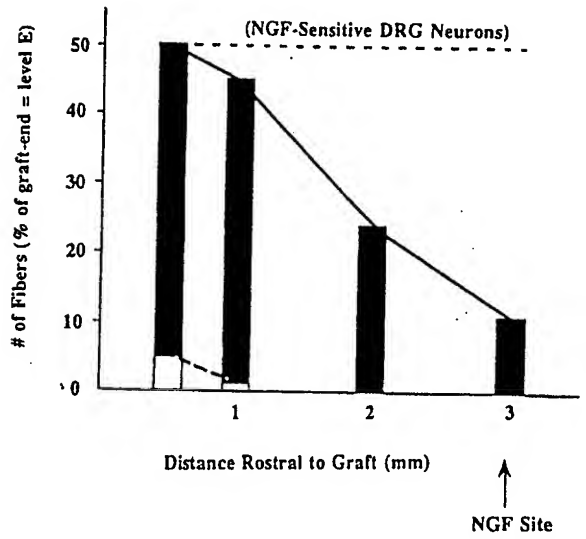

FIG. 5. Infused NGF promotes intracordal sensory regeneration (preliminary data). NGF was infused for 17 days, 3 mm rostral to the nerve bridge. CTB-positive fibers were counted at various distances rostral to the bridge end (level E).

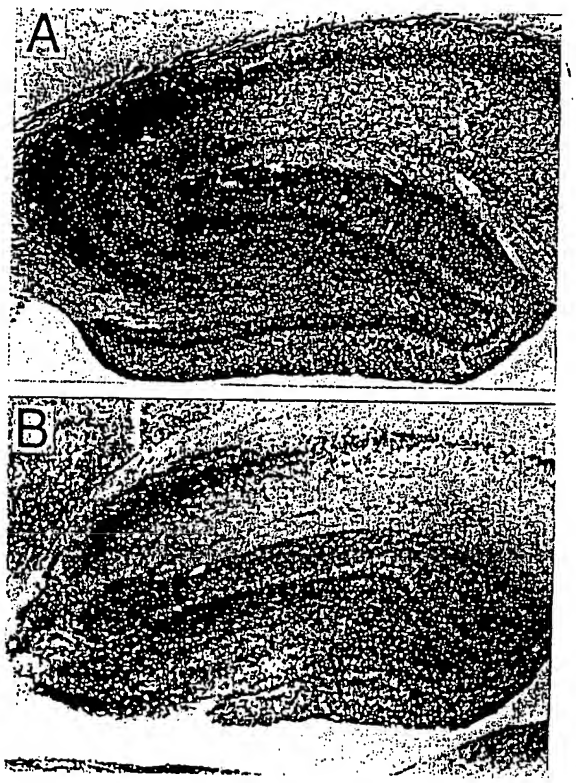

FIG. 6. Cholinergic axonal patterns in the hippocampal formation. (AChE staining in the dorsal HF.) A. Normal adult rat. B. Six months after fimbria-fornix lesion and bridge implantation. (Original magnification = ×30.)

must control them. One intriguing observation that may pertain to this question was provided by the septohippocampal cholinergic model, namely, a remarkable similarity between the cholinergic pattern in a normal adult HF and the pattern reestablished by regenerating cholinergic axons in the limited portion of HF that was reinnervated, that is, the most rostral 1.5 mm of the dorsal HF (Fig. 6) (Hagg et al., 1990a). Such a similarity

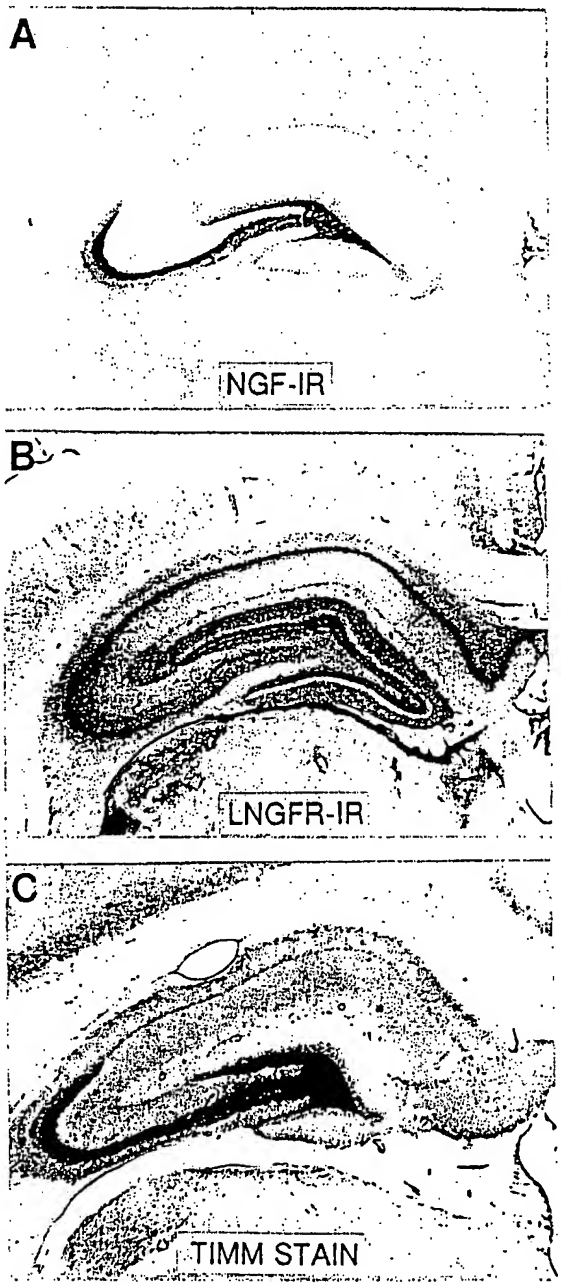

FIG. 7. Extrasomal NGF immunoreactivity (IR) in the mossy fiber region. The immunoreactive pattern for NGF (A) is different from that for low affinity NGF receptor, a marker for cholinergic afferents (B), but similar to a Timm staining pattern that selectively labels mossy fibers (C). (Original magnification = ×17.)

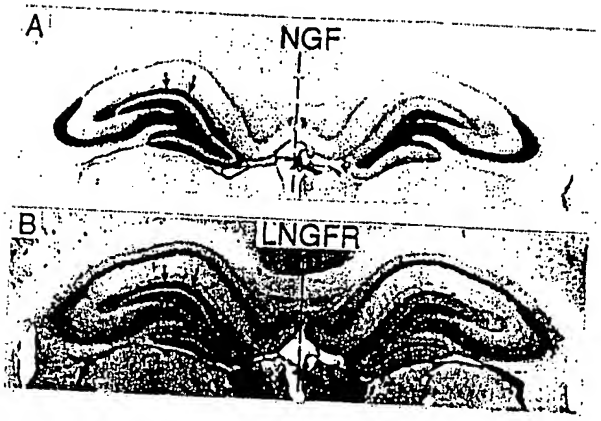

FIG. 8. Entorhinal cortex lesions induce a new region of NGF-IR in the dentate gyrus outer molecular layer. The new NGF band (A) is accompanied by a band of cholinergic terminal sprouting, revealed here by low affinity NGF receptor staining (B). Lesioned side indicated by arrows. (Original magnification = ×40.)

suggests that adult HF retains (or reestablishes after cholinergic deafferentation) a set of topographically organized cues that will direct, attract, or stop the regrowing cholinergic terminals. The recently acquired knowledge of the tropic properties of exogenous NGF in vivo (see preceding section) has prompted us to examine the distribution of endogenous NGF in the hippocampal formation by use of a potent anti-NGF polyclonal antibody and an immunostaining procedure selected to maximize protection of the NGF antigen and minimize nonantigenic IgG binding by adult rat brain tissue (Conner et al., 1992; Varon and Conner, 1993).

NGF immunoreactivity (NGF-IR) within cell bodies was found only in the basal forebrain cholinergic neurons that are expected users of NGF, and its rapid decline in those neurons after in vivo intracerebral treatments with colchicine (Conner and Varon, 1992) demonstrated the dynamic nature of acquisition and turnover of endogenous NGF by adult CNS neurons. CNS neurons recognized by others to contain NGF mRNA, and thus to be putative NGF producers, did not exhibit NGF-IR except after colchicine treatment (Conner and Varon, 1992), demonstrating that (1) they do not normally store the NGF they may be producing and (2) they distribute their NGF via colchicine-sensitive transport mechanisms, the blockade of which causes backup of NGF in their somata. Most pertinent to the present topic, and quite unexpected, was the observation of extrasomal NGF-IR in the hilus of the dentate gyrus and the CA3 and CA2 (but not the CA1) subfields of the hippocampus (Conner et al., 1992). The extrasomal NGF-IR (Fig. 7A) had sharply defined boundaries, suggestive of a firm association with local structures, differed from the HF pattern of cholinergic innervation (Fig. 7B) with regard to both subfields and laminar patterns, and was very similar to the pattern characteristic of mossy fibers (Fig. 7C), the granule cell axons projecting from dentate gyrus to the hippocampus proper. Similar mossy fiberlike regions (MF patches) of NGF-IR have been found to occur in brains of human and nonhuman primates (Mufson et al., 1993). Selective localization and extrasomal anchorage are two attributes that would be required if endogenous NGF were to serve as a marker for axonal routes and axonal endfields.

Further encouragement for such a speculation was obtained by exploiting the well-reported observation that removal of the entorhinal input to the HF creates a vacated synaptic field in the outer molecular layer (OML) of the ipsilateral dentate gyrus and that sprouting into the vacated zone will take place by adjacent afferents, including the cholinergic input through the fimbria-fornix (Lynch et al., 1972). We found (Conner et al., 1994) that an entorhinal lesion also results in the ipsilateral development of a new band of extrasomal NGF-IR, which at 8 days postlesion (Fig. 8A) is coincidental with the new band of cholinergic terminals detectable by their low affinity NGF receptor immunoreactivity (Fig. 8B) or by other specific cholinergic markers, such as AChE (data not shown). The development of the OML patch of NGF-IR precedes in time any detectable cholinergic sprouting and is demonstrably independent of the latter, since the patch occurs equally well in animals previously subjected to a fimbria-fornix transection (Fig. 9A,C,E), which removes the vast majority of cholinergic afferents to the HF (Fig. 9B,D,F). Thus, location and time course both propose that the new NGF-IR patch may be a required mediator of the lesion-induced cholinergic sprouting in the OML.

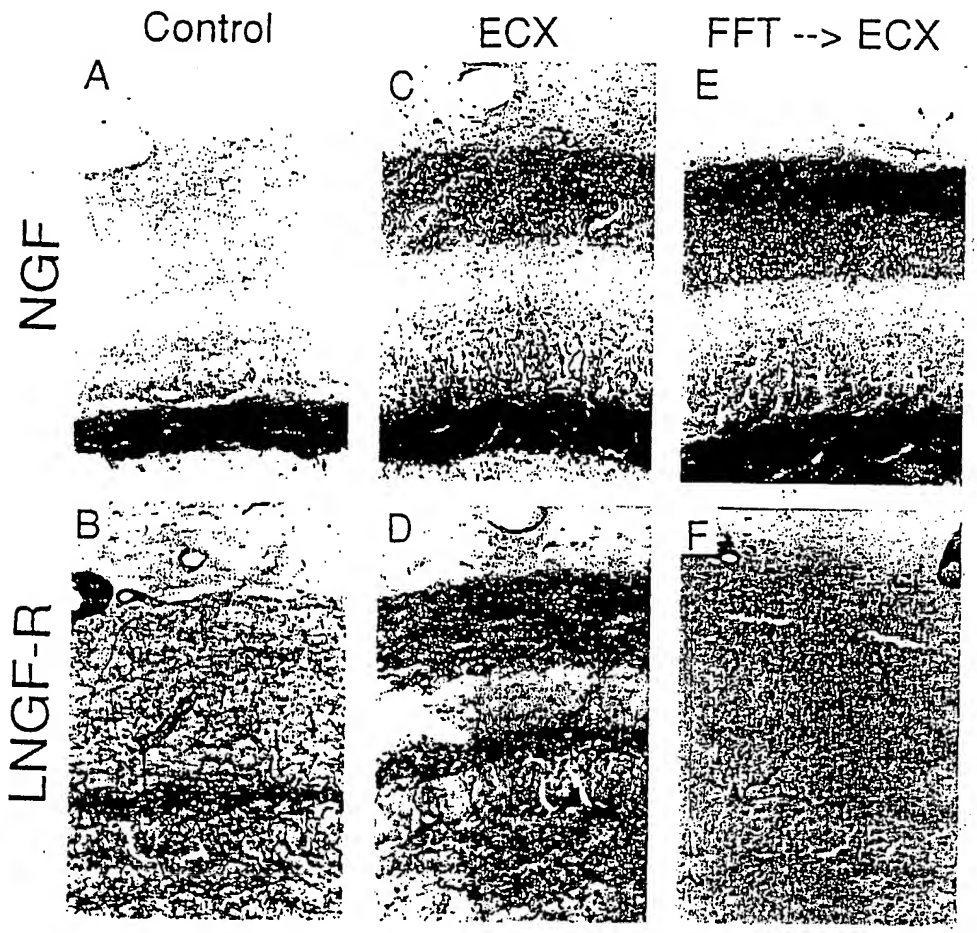

FIG. 9. The new NGF-IR band is independent of local cholinergic sprouting. The NGF-IR band appears in the outer molecular layer after entorhinal cortex lesion (C) even when the latter is preceded by a fimbria-fornix lesion (E). In contrast, the additional fimbria-fornix lesion prevents the appearance of a corresponding band of cholinergic terminals (F), which would otherwise be solicited by an entorhinal lesion (D). (A), (B): NGF and LNGF-R immunoreactivity in rats receiving neither the entorhinal (ECX) nor the fimbria-fornix (FFT) lesion. (Original magnification = ×160.)

To go beyond provocative correlations, one would require an actual demonstration that natural and lesion-induced NGF-IR patches could direct the growth of NGF-sensitive axons into HF tissue. Implants of superior cervical ganglia (SCG) into the cavity generated by a fimbria-fornix transection provided a convenient source of NGF-sensitive axons (Conner and Varon, 1993) with which to probe both the MF patch and the OML patch of NGF-IR. The mossy fiber pathway had already been reported to be selectively addressed by host sympathetic fibers but only following a fimbria-fornix transection (Loy and Moore, 1977; Crutcher et al., 1979). The new study, using DBH immunoreactivity to track sympathetic fibers, confirmed that implanted SCG would similarly innervate the mossy fiber pathway after (but not without) a fimbria-fornix transection (as shown by Bjorklund and Stenevi, 1977), establishing that the resulting cholinergic deafferentation of that HF region is crucial to the latter's suitability for new fiber invasion. The study also verified that the mossy fiber region addressed by the donor sympathetic axons did coincide with the previously recognized MF patch of extrasomal NGF-IR (Fig. 10A,C). Sympathetic fibers from the implanted SCG also reached into the outer molecular layers of the HF, but only when a prior entorhinal cortex lesion had both deafferented the region and created there the new OML patch of the NGF-IR (Fig. 10B,D).

DISCUSSION AND PROJECTIONS

The difficulty of adult CNS axons to regenerate into adult CNS tissue is increasingly perceived to lie more in an adverse CNS environment than in an intrinsic incompetence of the CNS neurons. From the earlier postulations of such a concept (Tello, 1911; Ramon y Cajal, 1928), one has advanced to more recent demonstrations that adult rat CNS axons will regrow in a peripheral nerve environment, whereas PNS axons will fail to do so in an optic (CNS) nerve (Aguayo et al., 1978). Furthermore, adult rat brain and cord neurons may grow axons for several centimeters into a sciatic nerve bridge yet fail to advance more than a few millimeters when faced again with CNS tissue (Aguayo, 1985; Aguayo et al., 1990). One speculation that links CNS tissue resistance to be penetrated and CNS axons reluctance to do so invokes the involvement of NTFs (Varon et al., 1984; Kromer and Cornbrooks, 1987; Hagg et al., 1993). Specifically, CNS axonal regrowth may require stimulation by appropriate NTFs that are not adequately available in the adult CNS tissue.

The recent work reviewed here has established that at least one neurotrophic factor, NGF, can stimulate the invasion of adult CNS tissue by adult CNS axons (cholinergic medial septum neurons) and adult PNS axons (sensory DRG neurons). It was also recognized that the neuritic responses to NGF depend on certain activation treatments, since only damaged MSC neurons are involved and only conditioned DRG neurons were adequately engaged. A second important feature brought forth by these studies is that NGF plays a tropic role in axonal regeneration and not only a trophic one. This feature has both negative and positive connotations. On the negative side, it cautions against inappropriate sites for exogenous NGF delivery and direction-distorting imbalances of NGF concentrations. On the positive side, it creates the possibility of coaxing NFG-sensitive axons to regrow into regions where they may eventually sort out to the appropriate functional positions. Finally, the recognition that endogenous NGF may occur in firm anchorage to discrete extrasomal locations raises the possibility that NGF (and possibly other NTFs) may also participate in the patterning of NGF-sensitive terminals. In this context, we are inclined to speculate that (1) in development, extrasomal NGF patches may form ahead of afferent ingrowth and disappear after the afferent pattern has become established, and (2) in the adult, extrasomal NGF would only be detectable in regions where local remodeling is likely to persist (e.g., the MF patch) or is newly solicited (e.g., the OML patch following entorhinal cortex lesions).

Thus far, the study of NTF involvement in adult CNS axonal regeneration has focused largely on NGF. An important question for future investigations is whether NGF is unique in those terms or whether other NTFs have similar axon-promoting competences, and, if so, which of the known NTFs do. NT3, like NGF a member

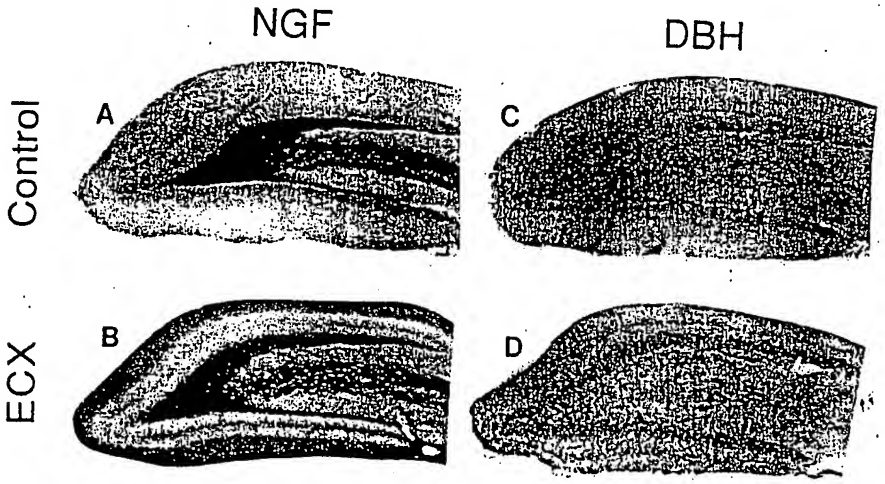

FIG. 10. Probing of NGF-IR regions with sympathetic axons from implanted superior cervical ganglia. A fimbria-fornix transection permits retention of NGF-IR in the mossy fiber region (A), which becomes a target for sympathetic (coarse DBH-immunostained) growing axons (C). Addition of an entorhinal cortex lesion elicits a second region of NGF-IR (B), which also solicits invasion by sympathetic fibers (D), (arrows). (Original magnification = ×30.)

of the neurotrophin family, has shown regeneration-promoting activity in vivo for adult rat corticospinal motor neurons (Schnell and Schwab, 1993) when administered in conjunction with antibodies to myelin-associated inhibitors of axonal regeneration (Schnell and Schwab, 1990; Cadelli and Schwab, 1991). Another factor, IL-1, a cytokine known to stimulate output of NTFs by glial cells (Lindholm et al., 1987; Carman-Krzan et al., 1991) also has been discussed in the context of axonal regeneration (Fagan and Gage, 1990). The field is barely tapped, and much progress is to be expected over the next few years.

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